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Development of a therapeutically important radiation induced promoter

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Radio-genetic therapy is a combination of radiation therapy and gene therapy that may solve some of the problems associated with conventional radiotherapy. A promoter responsive to radiation was obtained from a promoter library composed of DNA fragments created by linking the TATA box signal to randomly combined binding sequences of transcription factors that are reactive to radiation. Each promoter connected to the luciferase gene, was evaluated by luciferase expression enhancement in transfected cells after X-ray irradiation. The reactivity of the best promoter was improved by the random introduction of point mutations and the resultant promoter showed more than a 20-fold enhancement of the luciferase expression after X-ray irradiation at 10 Gy. The expression of downstream genes was also enhanced in stably transfected cells not only by X-rays but also by proton beam irradiation; and either enhancement was attenuated when an anti-oxidant was added, thus suggesting the involvement of oxidative stress in the promoter activation. Constructed promoters were also activated in tumors grown in mice. In addition, cell killing with the fcy::fur gene (a suicide gene converting 5-fluorocytosin to highly toxic 5-fluorouracil) increased dose-dependently with 5-fluorocytosin only after X-ray irradiation in vitro. These results suggest that promoters obtained through this method could be used for possible clinical applications.

Keywords: promoter, cis-element, gene expression, radiation, suicide gene therapy

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Introduction

Although radiation has been successfully applied for cancer therapy, it is often associated with harmful side effects. In addition, there are some cancers to which radiation therapy is being challenged. To address such problems, a new therapy called radio-genetic therapy, a combination of radiation therapy and gene therapy, is currently under investigation. It could be realized by employing a gene whose product modifies radiation effects on cancer tissue, or by employing a therapeutic gene whose expression is controlled by radiation. The latter combination could thus potentially allow for temporarily and spatially controlled therapy, in addition to lowering doses of either modality, presumably leading to a therapy which is more effective and less hazardous.

Cells start expressing genes for stress response and early growth response immediately after radiation stimulation. The promoters of such genes can be used as tools for controlling gene expression for radio-genetic therapy. For example, Hallahan and colleagues showed that the expression of a gene of interest could be controlled with the egr-1 gene promoter

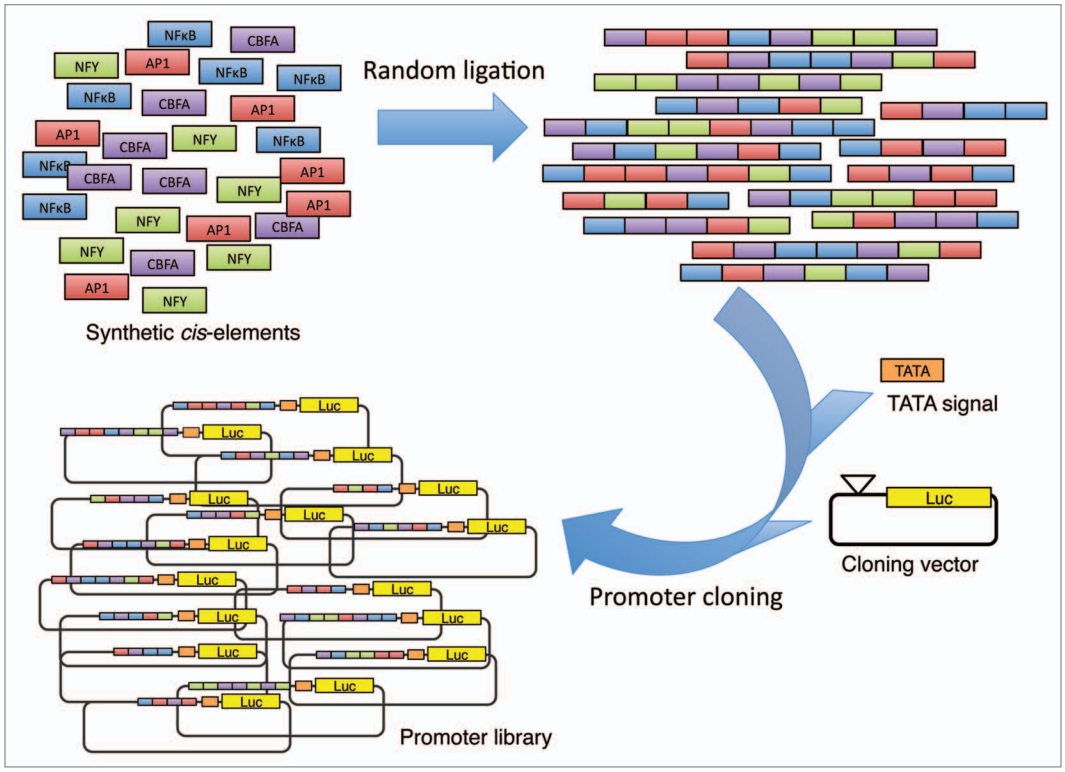

Figure 1. Schematic representation of the construction of a promoter library. Colored rectangles with their symbols (names of corresponding transcription factors) represent synthetic cis-elements. An orange rectangle with "TATA" represents a DNA fragment containing the TATA box sequence. A yellow rectangle with "Luc" represents the luciferase gene. "Cloning vector" in the figure is the pGL3-Control. Promoter fragments were cloned into plasmids upstream of the luciferase gene after removal of the SV40 promoter.

by radiation and that the size of a transplanted tumor in mice decreased after the TNFα gene was expressed under the control of the promoter by radiation.[1] However, naturally occurring promoters may have physiological limitations that may hamper their therapeutic use. Therefore, this study constructed artificial promoters that could be used for radiogenetic therapy (**Fig. 1**).

Construction of Artificial Promoters

There have been previous reports on the construction of artificial promoters. For instance, it is possible to bestow promoter activity on a DNA fragment that had no promoter activity by randomly introducing point mutations.[2] Li and colleagues constructed strong muscle specific promoters by randomly assembling cis-elements associated with the expression of muscle specific genes.[3] Marples et al. and Scott et al. constructed artificial promoters by combining CArG elements, a binding sequence of a transcription factor (cis-element) that activates the egr-1 gene promoter in response to radiation stimulation. They found that a promoter consisting of several copies of the CArG element showed a more sensitive response to radiation than the authentic one.[4,5] A promoter functions as a cis-element assembly and a cis-element consists of a nucleotide sequence, thus it would be possible to construct and improve promoters for certain genes of interest. However, it is often difficult to rationally design and construct improved promoters, since the binding of a transcription factor to its cis-element may depend on its context, including DNA conformations and epigenetic modifications.

Therefore, this study tried to construct radiation responsive promoters by randomly combining synthesized cis-elements of transcription factors that are activated by radiation stimulation. These were linked to a DNA fragment containing the TATA box signal. The radiation responsive promoters were intended to be constructed at a high rate so that a promoter with the desired property could be selected from a promoter library even with a limited number of promoter fragments. Nuclear factor-kappa B (NFκB), nuclear factor-Y (NF-Y), activator protein-1 (AP-1) and CArG element binding factor-A (CBF-A) were selected and the corresponding cis-elements were used to construct 11 DNA fragments that were subsequently cloned into pGL3-Control, a plasmid vector manufactured by Promega Corporation, upstream of the luciferase gene, in place of the SV40 promoter (the first library).

Each plasmid of the library was introduced into HeLa cells and the transiently transfected cells were subjected to a dual luciferase assay 6 h after X-ray irradiation of 10 Gy. Seven of the 11 clones (63.6%) exhibited significantly increased luciferase activity in comparison to corresponding transfected cells without X-ray irradiation ($p < 0.05$). The clone 11 promoter, which showed a 5.3-fold expression increase, was found to be the best in the library.

The study then attempted to improve the reactivity of the clone 11 promoter to radiation by randomly introducing point mutations. This method was expected to improve the reactivity because a previous study confirmed that it was possible to improve a promoter property by introducing random point mutations.[3] The clone 11 promoter was PCR-amplified in a reaction mixture containing Mn^{2+} in addition to Mg^{2+} to interfere with the fidelity of Taq polymerase, thus introducing random point mutations. The results confirmed about 1% of point mutations were introduced in DNA fragments after PCR amplification in a reaction mixture containing 0.75 mM Mn^{2+}. The best promoter was selected using the enhancement ratio of luciferase expression after irradiation among the mutant fragments generated by the PCR reaction and then subjected that promoter to a further round of PCR in the same reaction mixture. The results showed that the clone 11-9-37 promoter was the best promoter of all the generated mutant promoters.[6]

This promoter drove the expression of the luciferase gene by more than 20-fold at 6 h after X-ray irradiation at 10 Gy, in comparison to that without X-ray irradiation. However, the reactivity appeared to be specific to HeLa cells, showing no luciferase expression enhancement in other cancer cell lines. It is likely that this may be because a different set of the transcription factors are activated in different cell lines. Actually, a responsive promoter to radiation was successfully obtained by a similar method using a different set of cis-elements of transcription factors in a prostate cancer cell line in which clone 11-9-37 promoter did not significantly enhance luciferase expression in response to radiation.[7]

The effectiveness of this method was demonstrated by constructing another promoter library composed of 62 promoter fragments using cis-elements of the same set of transcription factors (the second library). The results showed that 57 promoters significantly enhanced luciferase expression after X-ray irradiation at 10 Gy (91.9%) and that three of these promoters, clones 831, 843 and 848, enhanced the expression of the luciferase gene more than 10-fold 6 h after X-ray irradiation at 10 Gy. These results again indicate the efficacy of this methodology, easily yielding promoters responsive to stimulation at a high ratio.

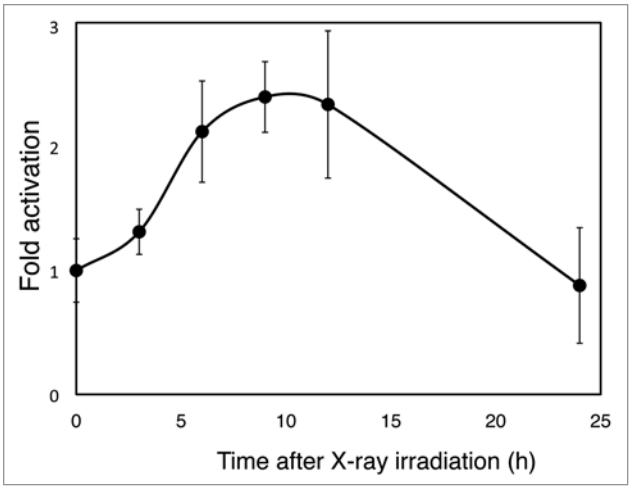

Figure 2. Kinetics of luciferase activity expressed by the clone 11-9-37 promoter after 2 Gy X-ray irradiation. Bars represent standard deviations (n = 4).

Although radiation-responsive promoters were obtained out of a few libraries that were constructed in a similar manner, there was no promoter showing enhancement far over those of these three promoters (about 10-fold enhancement after 10 Gy X-ray irradiation), possibly representing an upper limit for the method. It may therefore be necessary to screen several orders of magnitude more promoter fragments and/or improve the reactivity of promoters obtained by this method in order to obtain much more reactive promoters. It may be necessary to introduce positive screening methods, such as the ones shown to be effective by Dai et al. and Sclabach et al., since this method does not deal with screening much higher numbers of promoters.[8,9] A much better promoter may be obtained by using new methods to improve promoter reactivity including random introduction of point mutations as described above, and DNA shuffling.[10]

Properties of Artificial Promoters in Stably Transfected Cells

A transfection system mediated by a recombinant retrovirus vector was established to further investigate the properties of the constructed promoters. Some promoter reactions in stably transfected cells were somewhat different in comparison to those observed in transiently transfected cells. The ratios of luciferase expression enhancement by the clone 831, 843 and 848 promoters were attenuated in comparison to those observed with transient transfection experiments, probably due to higher basal activities (activity without stimulation). The HeLa/Ret-37-luc cell line was established by the stable introduction of the luciferase gene under the control of clone 11-9-37 promoter with recombinant retrovirus vector. The peak enhancement ratio in these cells after X-ray irradiation at 10 Gy was unchanged in comparison to that observed in transiently transfected cells at 24 h after irradiation, and the luciferase activity subsided to the level of that without radiation, showing a less sustained effect than that in transiently transfected cells. Clone 11-9-37 reacted to 2 Gy X-ray, an equivalent to a single radiation dose of fractionated exposure that is usually applied for cancer therapy. Luciferase activity increased to 2.4-fold ($p < 0.01$) at its peak 9 h after X-ray irradiation and then decreased thereafter to the level of that without radiation 24 h after irradiation (**Fig. 2**), suggesting the possibility to obtain a clinically applicable promoter by this method. This promoter could enhance expression of other genes including the enhanced green fluorescent protein.

Proton beam irradiation facilities have been established for cancer therapy to treat the increasing numbers of cancer patients.

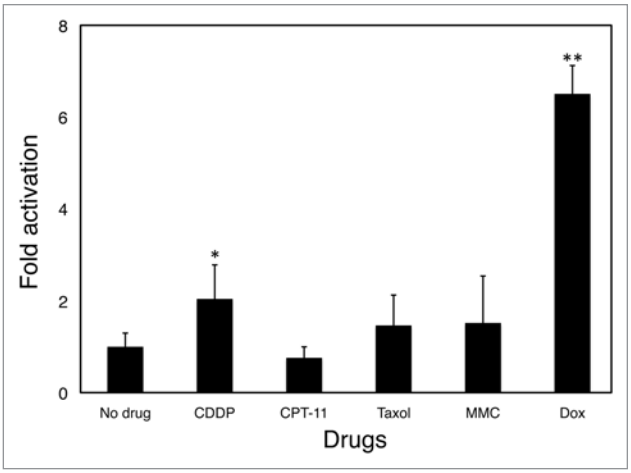

Figure 3. Enhancement of luciferase activity by the clone 11 promoter activated with anti-cancer drugs. A drug was dissolved in medium at a concentration of 5 μM and cells were incubated in the medium with the drug for 30 min. The cells were washed three times in fresh medium and luciferase activity was evaluated by a dual luciferase assay 6 h after drug treatment. Bars represent standard deviations (n = 4). No drug, mock treatment; CDDP, cisplatin; CPT-11, irinotecan; Taxol, paclitaxel; MMC, mitomycin C; Dox, doxorubicin.

The W-MAST synchrotron is a device available in the Wakasa Wan Energy Research Center that is designed for cancer therapy, which can generate proton beams up to 200 MeV. Although a proton beam, a flow of particles with mass, has different physical properties from a light beam including X-rays, its biological effects are similar to those of X-rays. For example, the RBE (relative biological effectiveness) of a proton beam is 1.11, calculated based on observations of mouse intestinal villi damage after irradiation with either proton beam or X-ray.[11] Further experiments examined whether the promoters constructed in this study were responsive to proton beams. Four recombinant HeLa cells lines stably transfected with a gene cassette composed of one of the four highly reactive promoters and the luciferase gene were irradiated with a 10 Gy proton beam. Even though the cell lines received a dose of proton beam identical to that of X-ray, lower enhancement of luciferase expression was observed, though luciferase activities in all the cell lines significantly increased after proton beam irradiation. Proton beam and X-ray generate differing amounts of reactive oxygen species.[12] Such a physicochemical difference between the two types of radiation might affect their biological effects, resulting in different responses by the promoters. Though the ratios of increase were lower after proton beam irradiation than those observed after X-ray irradiation, the relative enhancement ratios among the recombinant cell lines were similar, suggesting a similar underlying mechanism of promoter activation by proton beams and by X-rays.

It is widely accepted that stimulation by radiation brings cells to a state of oxidative stress.[13] In addition, some transcription factors including NFκB and AP-1 that were chosen to construct the current promoters, are activated in response to oxidative stress conditions.[14] Therefore, it would be reasonable to hypothesize that oxidative stress may be involved in the activation of clone 11-9-37 promoter. The effects of adding hydroxyl radical scavengers including dimethyl sulfoxide (DMSO) and D-mannitol to HeLa/Ret-37-luc culture on the enhancement of luciferase activity by irradiation by either proton beam or X-ray was investigated. The enhancement of luciferase activities by X-ray decreased to 64% with 70 mM DMSO and to 65% with 100 mM D-mannitol. In addition, the enhancement by proton beams decreased similarly. These results suggest that the activation of clone 11-9-37 involves oxidative stress caused by radiation stimulation. **Figure 3** shows that the clone 11 promoter is also activated by stimulation with doxorubicin, an anticancer agent, by increasing the expression of a gene connected downstream that may be due to oxidative stress since doxorubicin has also been reported to cause oxidative stress.[15] On the other hand, other drugs such as mitomycin C do not activate clone 11 despite being an oxidative stress facilitator.[16] These results suggest that although oxidative stress is involved in activation of clone 11 promoter, oxidative stress alone may not be sufficient and that there must be other factors involved in the activation process. For example, interaction of drugs with DNA could be involved in the process since doxorubicin and cisplatin are known to interact with DNA, although it might not be so simple, since interactions of doxorubicin and cisplatin with DNA differ in detail. In addition, mitomycin C is also known to interact with DNA.[17]

A Potential Role for the Constructed Promoters in Clinical Applications

Nude mice were used as model animals to evaluate the applicability of promoters in the living body. HeLa/Ret-37-luc, HeLa/Ret-31-luc (HeLa cells retrovirally introduced with a gene cassette of clone 831 promoter and the luciferase gene) and HeLa/Ret-SV-luc (HeLa cells retrovirally introduced with a gene cassette of SV40 promoter and the luciferase gene) were subcutaneously injected on both flanks of a mouse and allowed to grow into tumors. One of the tumors in each mouse was irradiated with X-ray at 10 or 15 Gy while leaving the tumor on the other flank as the unirradiated control. The expression of luciferase in tumors was evaluated 9 h after X-ray irradiation. The HeLa/Ret-37-luc tumors showed a 2.3-fold ($p < 0.05$) and 2.7-fold ($p < 0.05$) increase in photon signals in comparison to the unirradiated controls 9 h after 10 and 15 Gy X-ray irradiation, respectively. The HeLa/Ret-31-luc tumors showed a 1.7-fold ($p < 0.05$) and 2.3-fold ($p < 0.05$) increase in photon signals 9 h after 10 and 15 Gy X-ray irradiation, respectively, while the tumors with the SV40 promoter showed no significant increase of photon signals after X-ray irradiation at 10 Gy. Although the enhancement ratios were attenuated in comparison with those observed in vitro,

Table 1. Summaries of constructed promoter charateristics

Promoter	Library derived from	Improvement	Maximum enhancement ratio[1]		In vivo enhancement[2]	In vitro suicide gene therapy[3]	GenBank accession#
			Transient transfection	Stable transfection			
Clone 11	First (11 clones)	ND[4]	5.6	ND	ND	ND	EF536080
Clone 11–9-37	First (11 clones)	Two rounds of epPCR	21.6	18.8	Yes[5]	Yes[6]	EF536082
Clone 831	Second (62 clones)	ND	11.2	6.5	Yes	ND	HQ542862
Clone 843	Second (62 clones)	BD	13.5	2.9	ND	ND	HQ418223
Clone 848	Second (62 clones)	ND	11	2.9	ND	ND	HQ418224

[1]A ratio of luciferase activity expressed by a promoter 6 h after X-ray irradiation at 10 Gy to that by the same promoter without X-ray irradiation; [2]Evaluated in a tumor tissue of HeLa/Ret-37-luc or HeLa/Ret-31-luc formed on a mouse; [3]With the fcy::fur gene and 5-FC; [4]Not done (ND); [5]Detected significantly increased luciferase activity evaluated by photon generation of bioluminescence after X-ray irradiation at 10 or 15 Gy; [6]Detected significantly decreased cell survival dose-dependently with 5-FC only after X-ray irradiation at 10 Gy.

there were significant increases of photon generation after X-ray irradiation in vivo. The reason for the attenuation currently remains unknown. The basal activity levels may be higher in vivo, possibly due to activation of transcription factors that bind to the constructed promoters that may have been induced by biologically active factors in the blood such as hormones and cytokines.

The fcy::fur gene, a fusion gene between the yeast fcy1 and fur1 genes,[18] whose product converts 5-fluorocytosin (5-FC) to highly toxic 5-fluorouracil (5-FU), was then employed to execute an in vitro suicide gene therapy simulation study. This cell line increased the amount of fcy::fur gene product in response to X-ray irradiation. HeLa/Ret-37-Fcy::Fur cells were cultured in medium containing 5-FC. The cell viability decreased dose-dependently with 5-FC only after X-ray irradiation. The decreased viability was thought to be caused by X-ray facilitated conversion of 5-FC to 5-FU by the fcy::fur gene product increase, since a similar phenomenon was not observed with HeLa/Ret-37-luc. However, the cell viability significantly decreased when high concentration of 5-FC was added, even without X-ray irradiation, probably due to leaky expression of the fcy::fur gene product in HeLa/Ret-37-Fcy::Fur caused by a relatively high basal activity of clone 11-9-37 promoter.

These results suggest that although it is difficult to apply clone 11-9-37 itself for clinical use, the current method could possibly produce a clinically applicable promoter.

Conclusion and Perspective

This study showed that promoters constructed using the current method were activated in response to proton beam in addition to X-ray irradiation, thus controlling expression of a gene of interest connected downstream. Moreover, the results confirmed that such a promoter could be applied for controlling gene expression in vivo and suicide gene therapy in vitro. The characteristics of the constructed promoters are summarized in **Table 1**.

The biggest problem is the relatively high basal activity of promoters in vivo observed in the absence of stimulation. Experiments are now underway to introduce a system to minimize the expression of a gene of interest without radiation stimulation. For instance, the identification of a transcription factor that is creating higher basal activity in vivo will allow the reconstruction of promoters using a set of cis-elements of transcription factors without that particular transcription factor. In addition, the introduction of target sequence motifs of microRNAs whose expression decreases in response to radiation stimulation into the 3'-untranslated region of the gene of interest may suppress gene expression when no stimulation is provided.

The current method to construct radiation responsive promoters is simple and efficient. This method may also be used to obtain promoters responsive to stimulation other than radiation. Other studies actually obtained promoters responsive to stimulation by low intensity ultrasound[19,20] and an anti-cancer drug[21] from a library using a method that was similar to that shown in this study. Therefore, it is reasonable to consider that promoters obtained by this method could be utilized not only for radio-genetic therapy but for a wide range of biomedical applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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